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萱草花粉类动力蛋白生物物理学及药理学性质*

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摘要: 利用 FPLC 技术从萱草花粉中鉴定并纯化了动力蛋白, 研究了它的酶学性质及部分生物化学性质。结果如下: 纯化的类动力蛋白分子量为 100 kD, 等电点 pI = 6.15 和 6.80。在 280 nm 波长激发下, 最大的荧光发射波长是 346 nm。荧光光谱分析结合紫外吸收光谱及导数光谱分析推断它含有色氨酸和酪氨酸残基。药理学性质研究表明巯基可能在酶的活性中心起重要作用。

关键词: 类动力蛋白; 分子马达; 微管; 花粉

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Biophysical and Pharmacological Characterization of a Dynamin-like Protein from Day-lily (*Hemerocallis fulva*, Liliaceae) Pollens

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Abstract: A novel motor protein, dynamin-like protein from day lily, was purified and identified by FPLC. Here we report its biochemical characterization. The molecular weight of the dynamin-like protein is 100 kD on SDS-PAGE. Isoelectric points are about 6.15 and 6.80. The fluorescence emission wave length of dynamin-like protein is 346 nm by excitation at 280 nm. Through fluorescence spectra analysis, ultraviolet absorption spectrum and derivative spectrum, we conclude that it contains tryptophan and tyrosine residues. Pharmacological study indicates that mercapto may play an important role in enzyme activity of dynamin-like protein.

Key words: Dynamin-like protein; Molecular motor; Microtubule; Pollen

Movement is an essential function of all the cells and the basis of all life activities. Long before, cell biologists have speculated that all activities in the cell were the result of the functions of so-called mechanoenzymes. However, none of these mechanoenzymes has been identified. It is well known that myosin is the movement motor of the microfilament system. Kinesin and cytoplasmic dynein were identified as motor proteins (Vale *et al*. 1985), which were regarded as the enzymes to couple the hydrolysis of nucleotide and movements of the microtubules. Recently, the study on motor protein, which plays a significant role in eukaryotic cells, has been one of the focuses in modern cell biology and biochemistry (Skoutias and Scholey, 1993; Wu and Yan, 2002). The protein that was found in bovine brain tissue with the molecular weight 100 kD was named dynamin-like protein (Shpetner and Vallee, 1989), which GTPase activity was shown to be high, while ATPase activity to be low. However they could be also stimulated 1.6-fold by microtubules. This was

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the third microtubule motor protein that was importance to microtubule slippage and transport of the vesicles, succeeding to kinesin and cytoplasmic dynein which were found in bovine brain tissue .

Previously, all the reports on dynamin-like protein were from animal materials . While Wu and Yan (2002) recently have firstly isolated a dynamin-like protein from plant day-lily which is similar to nerve cell protein . If like this, it may prove that the plant cell and animal cell has the same mechanism in the energy transmission and the information transmission . The research proved that this dynamin-like protein, which can take the immuno-cross reaction with bovine brain protein antibody, has both GTPase and ATPase activity, but the former one is far higher than the latter one . And the investigations on the biophysical and the pharmacological properties of day-lily′s dynamin-like protein are reported here .

1 Materials and Methods

1.1 Materials

Day-lily (*Hemerocallis fulva* L .) pollens were obtained from Institute of Medicinal Plant, Chinese Academy of Medical Sciences . The anthers were irradiated in 60W lamp to dehisce . Then pollens were selected by shakesieve . The pollens were dried and stored at -80℃ .

1.2 Separation and purification

25 g day-lily pollens were triturated in ice water with 60 ml -100 ml extract buffer (100 mmol/L PEPES, pH 6.9 including 1 mmol/L $MgCl_2$, 1 mmol/L EGTA, 1 mmol/L DTT, 1mmol/L PMSF, 50 μg/ml TAME, 50 μg/ml TPCK 和 0.5% PVP) The extracted liquid was isolated in 30%-80% fraction of ammonium sulfate precipitation . Then supernatant was treated with DEAE-Sephadex A50 column chromatography with 0.5 mol/L KCl as eluent . The liquid was collected at 280 nm peak . After centrifugation at 15 000 r/min for 15 min at 4℃, the supernatant was purified by FPLC-Mono Q ion exchange chromatography using 0 -600 mol/L continuous concentration gradient KCl as eluent .

1.3 Determination of themolecular weight of the dynamin-like protein

SDS-PAGE was performed according to the Laemmli (1970) method, with a concentration of 4.8% for the separating gel and a concentration of 10% for the concentrating gel . Gels were stained with silver nitrate . The molecular weight of the dynamin-like protein was determined by the relationship between the logarithm molecular weight and the electrophoretic mobility .

1.4 Determination of isoelectric point

The measurement of isoelectric point of the protein was determined with phast system IEF (Pharmacia) . The sample was loaded 0.4 μl . PhastGel™ (5%T, 3%C), pI 3.0-9.0, were used as prepared gels and stained with silver nitrate . The standard was from Pharmacia Company .

1.5 Fluorescence spectra analysis

The fluorescence emission wave length of dynamin-like protein is measured with a F-4010 fluorescence spectrophotometer (Hitachi) .

1.6 Ultraviolet absorption spectrum and derivative spectrum analysis

The Ultraviolet absorption spectrum and derivative spectrum are measured with a Beckman DU-7000 spectrophotometer .

1.7 Determination by the Pharmacology

In the determination of Pharmacological character of day-lily dynamin-like protein, various inhibitors or activating agent, such as 100 μmol/L Oligomycin, 100 μmol/L Ouabain, 2.4 mmol/L NaF, 2.4 mmol/L NaF^+ and 0.1 mmol/L $AlCl_3$, 0.25% TritonX-100, 1 mmol/L PCMS, 0.1 mmol/L NEM, 1 mmol/L NEM, were added in respectively, compared with the blank sample in which no inhibitor was added . The method given by Gonzalez-Romo (1992) was used to determine ATPase activity . The enzyme activity unit is nmol $Pi \cdot min^{-1} \cdot mg^{-1} Pr$.

1.8 Determination of protein concentration

The protein concentration was measured by UV-240 spectrophotometry (Shimadzu), BSA as a standard protein (Bradford, 1976) .

2 Results

2.1 Separation and purification

Ammonium sulphate fractional precipitation, DEAE-Sephadex A50 column chromatography and FPLC-Mono Q ion exchange chromatography could purify the dynamin-like protein from the extract of day-lily pollens and increase its purity by 86 times (Table 1) .

2.2 Determination of the molecular weight

Results of SDS-PAGE that stained by silver nitrate are presented in Fig . 1 . The molecular weight of the dynamin-like protein is 100 kD estimated by the relationship between the logarithm molecular weight and the electrophoretic mobility, which is similar to the animal dynamin-like protein (Shpetner and Scholy, 1993) .

2.3 Determination of isoelectric point

Isolectric points of the plant dynamin-like protein are 6.80 and 6.15 (Fig . 2) which may be resulted from the composition and structure of the amino acids . It shows that the dynamin-like protein is electrically neutral in both buffer solutions pH 6.80 and pH 6.15 .

2.4 Fluorescence spectra analysis

Table 1 Purification of dynamin-like protein from day-lily pollen

Purification steps	Total protein	Specific activity (nmol Pi . mg^{-1} min^{-1} Pr .)	Purified fold
Crude extracts	669.6	6.8	1
$(NH_4)_2SO_4$ precipitate	238.4	17.6	2.5
DEAE-Sephadex A_{50}	8.7	272.4	40.1
FPLC-Mono Q	0.84	585.1	86

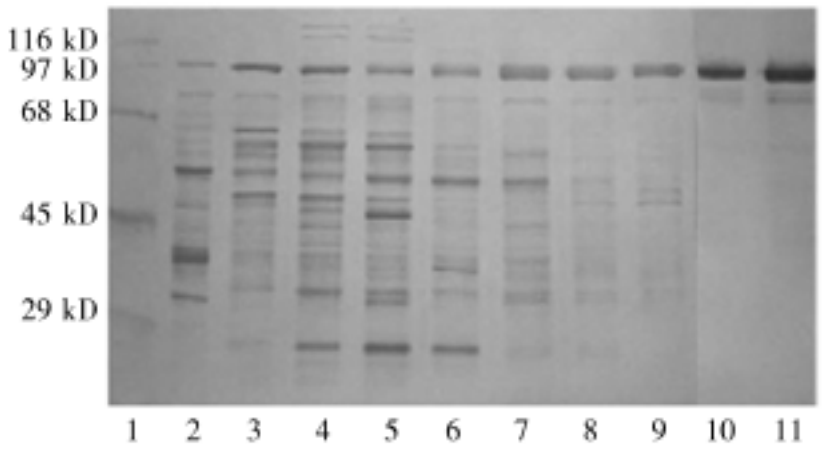

Fig . 1　SDS-PAGE of plant dynamin-like protein by FPLC-Mono column

lane 1: Sigma Mr . Standard

lane 2: Crude dynamin-like protein from $(NH_4)_2SO_4$ precipitate

lane 3: Purified dynamin-like protein from DEAE A_{50}

lane 4-11: Different fractions from Mono Q column

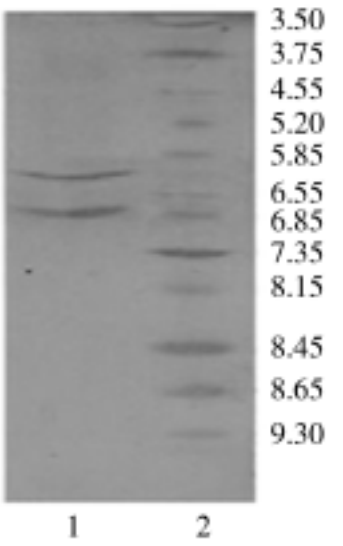

Fig . 2　Determination of PI volue of dynamin-like protein from day lily pollen by IEF-PAGE

lane 1: Purified dynamin-like protein; lane 2: Standard Marker proteins .

The fluorescence analysis method is usually used to investigate the structure of the protein that based on its self-fluorescence . The fluorescence emission wave length of dynamin-like protein is 346 nm by excitation at 280 nm (Fig . 3), indicating it may contain the tryptophan residue .

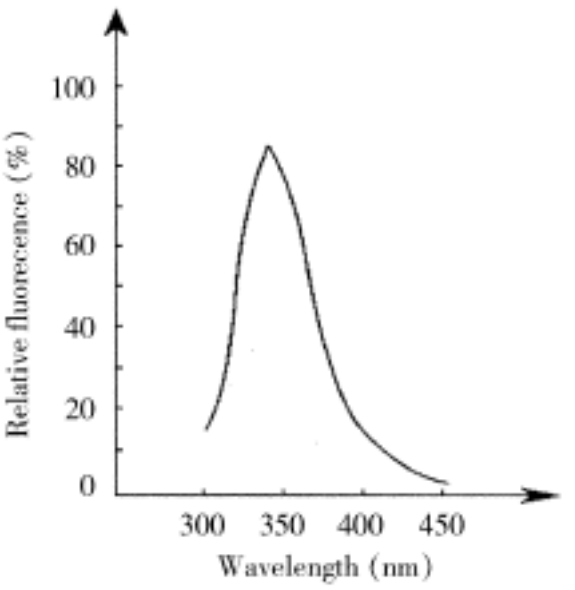

Fig . 3　Fluorescence spectrum of dynamin-like protein

2.5　Analysis of ultraviolet absorption spectrum and derivative spectrum

The ultraviolet absorption spectrum of dynamin-like protein was shown in Fig . 4 . Its maximal absorbing wave is 276.2 nm (Fig . 4A), contributed by indole cycle in tryptophan and hydroxybenzene in tyrosine . Derivative spectrum is sensitive to the intensity transformed by length of the wave, especially producing strong signals in the slope or bent site so as to provide more message and complicated structure, which is meaningful to the inspection of spectrum curve obliterated by broad band spectrum or non-structure background spectrum . We have inspected the first, second and third rank derivative spectrum of dynamin-like protein, shown in Fig . 4B, C, D . It represents an absorption peak in ultraviolet absorption spectrum (Fig . 4A), while it represents four absorption peak in the first rank of derivative spectrum (Fig . 4B), which presents more complicated structure (Fig . 4C, D) .

2.6　Pharmacological character of day-lily dynamin-like protein

The effect of some kinds of inhibitor and the activating agent on dynamin-like protein ATPase enzymatic activity is listed in Table 2, indicating that Triton X-100 has the activity function slightly but Oligomycin is insensitive to it . NaF、Ouabain presents a certain kind of restraint feature, while $AlCl_3$ + NaF and PCMS strongly suppress its activity . NEM can suppress 59% of them, which is similar to the study result on bovine brain protein (Shpetner and Vallee, 1992) . Wu and Yan (2002) also obtained the same trend in the inspection of GTPase activity of dynamin-like protein . NEM can only inhibit kinesin as concentrations above 2 mM, but suppress dynamin-like protein at concentration as low as 0.1 mM . The inhibition becomes stronger when the concentration of NEM becomes higher . The mercapto inhibitor, NEM and PCMS, which can strongly inhibit the enzyme activity, indicates that mercapto was presumed to play an important role in the enzyme′s active site .

3　Discussions

In all plant microtube motor proteins, only kinesin was identified (Tiezzi *et al* . 1992), so we anticipated that all the movement of cell would have the corresponding motor . If microtubes exist in the cell, there must be molecular motors reacting with the microtubes in it . For microtubes participate in many life movements, the quantity of the corresponding motor protein should be enough to show adequately the function of

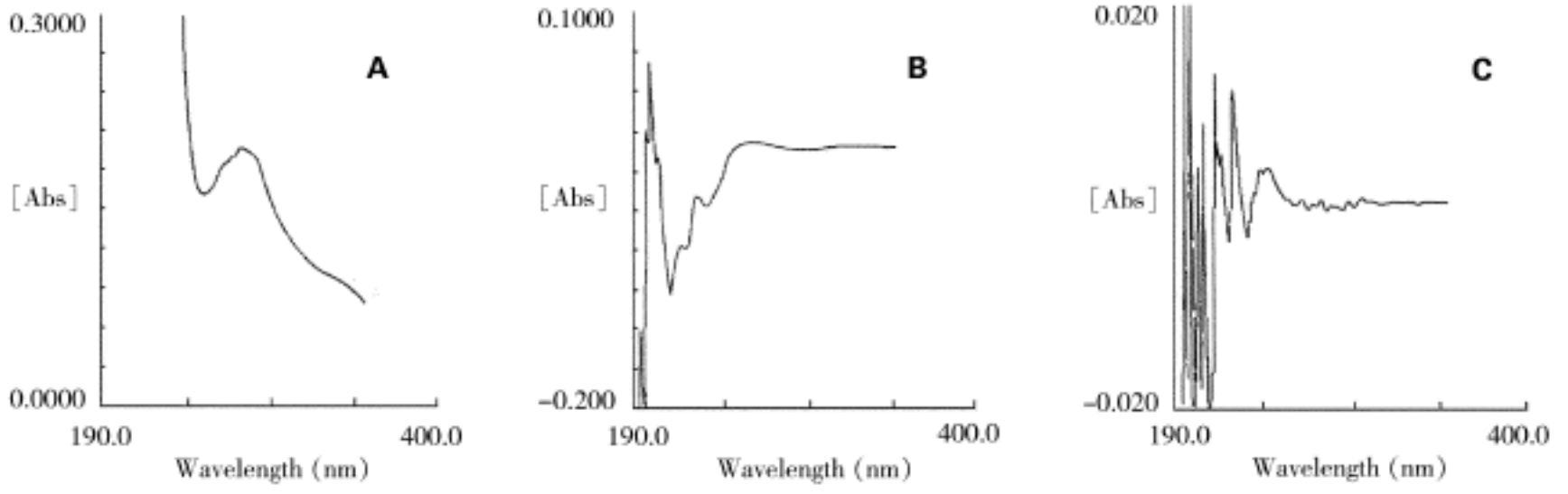

Fig . 4 Ultraviolet absorption and derivative spectrum of dynamin-like protem trom day lily pollen
A . Ultraviolet absorption spectrum; B . 1st derivative spectrum; C . 2nd derivative spectrum; D . 3nd derivative spectrum

Table 2 Pharmacological characterization of dynamin-like protein

Inhibitor	ATPase activity (bnol Pi .min^{-1} .mg^{-1} .Pr .)	% control
100 μmol/L Oligomycin	189.55	92.96
100 μmol/L Ouabain	99.08	48.59
2.4 mmol/L NaF	101.11	49.59
2.4 mmol/L Naf + 0.1 mmol/L $AlCl_3$	30.09	17.70
0.25% Triton X-100	220.21	108.00
1 mmol/L PCMS	30.08	14.75
0.1 mmol/L NEM	85.50	41.90
1 mmol/L NEM	113.82	55.82
No inhibitor	203.90	100

microtube system . In the investigation of Yan and Wu (1993), dynamin-like protein was found in myxomycete and towel gourd pollen . Wu and Yan (2002) reported several properties of day-lily pollen dynamin-like protein, it is obviously proved that its properties is very similar to those of animal dynamin on some aspects such as molecular weight, substrate pharmacological character on GTPase . Here we continue to reveal the biophysical and the pharmacological properties of dynamin-like protein . The molecular weight of dynamin-like protein of purified day-lily pollen is 100 kD (Fig . 1) . The result is consistent with that of bovine dynamin (Shpetner and Vallee, 1989) . Pharmacological character on ATPase (Table 2) is very silimar to pharmacological character on GTPase (Wu and Yan, 2002) . Through fluorescence spectra analysis and ultraviolet absorption spectrum, we can conclude that it contains tryptophan base and tyrosine base (Fig . 3, Fig . 4A) . And derivative spectrum gives the finer structure of dynamin-like protein (Fig . 4B, C, D) . The results are not referred in animal dynamin . On our study Oligomycin, inhibiting the ATPase in mitochondrion membrane, was not the inhibitor to dynamin-like protein (Table 2), indicating it is not only energy supply but also can join the cell life movement involving particulate transportation, endocytic activity and signal transudation . The further study on the orientation and physical function of plant dynamin-like protein is expected earnestly . Whatever it will be molecular motor or one member of G-protein, the significance of it in cell is undoubted .

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